Semisynthetic combinatorial antibody libraries: A chemical solution to the diversity problem

(surface expression/random mutagenesis/naïve antibody repertoire/catalytic antibodies/human antibodies)

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ABSTRACT The properties of naiveté and large diversity are considered to be essential starting features for combinatorial antibody libraries that eschew immunization by evolution *in vitro*. We have prepared large libraries with such properties by using random oligonucleotide synthesis, which has the potential to create approximately 10^{20} complementarity-determining regions for antibody heavy chains. When combined with light chains and expressed on phage surfaces, high-affinity antibodies could be selected from 5.0×10^7 *Escherichia coli* transformants. Remarkably, antibodies selected only for binding displayed both general structural features known to be important in nature's own antibodies and specific consensus sequences thought to be critical for interaction with the hapten against which the library was selected. Semisynthetic and ultimately totally synthetic combinatorial libraries when coupled with mutation and selection procedures should replace immunization for generation of reagent, therapeutic, and catalytic antibodies.

The time-honored process of immunization to produce antibodies is rapidly being replaced by the *in vitro* generation of combinatorial antibody libraries (1). The ultimate success of this method depends on the extent to which nature's mechanisms can be copied and/or improved. The essence of the process by which antibodies are generated can be distilled into four major issues—most of which have been duplicated by methods already in use for the generation of combinatorial antibody libraries. First, the generation of antibodies is at every level a combinatorial process (2). The genetics of antibody diversity allows for combinatorial joining of nucleic acid segments and the structure of the protein molecule provides for the combinatorial association in three-dimensional space of six peptide loops. The peptide loops are distributed among two protein chains that can randomly associate with each other. Second, antigen recognition and cell replication are linked. This linkage ensures that the specific expansion of clones reactive with the antigen and is essential to the third feature of the process, which is that of somatic refinement though mutation and selection. By their very nature, combinatorial antibody libraries in which very large numbers of different heavy and light chains are randomly combined duplicate many aspects of the mechanisms used by the natural immune system. The incorporation of these libraries into surface display vectors provides the essential link between recognition and replication and allows for the process of B-cell selection to be mimicked (3). Although these features are important, the fourth element, which is that the starting repertoire is naive, is of equal or greater significance. This requirement leads to the critical question of where one gets the genetic material to generate combinatorial libraries. The central idea is that, unless one starts with a naive library, one is engaged in the formidable task of remodeling proteins that are already highly evolved to interact with another molecule. Consider, for example, the task of converting an antibody evolved to bury a small organic ligand deep in the binding pocket into one that binds another protein where the most effective interaction may involve a rather flat approximation of the protein surfaces with binding contacts distributed over a large area. An ideal way of obtaining a totally naive combinatorial library is to chemically synthesize the six complementarity-determining regions (CDRs) in a random way and present these synthetic CDRs on a varied framework region (FR). A first approximation would be to randomize the heavy chain CDR3 (HCDR3) since it generally contributes most in terms of antigen-binding contacts and diversity.

Herein we report an expansion of the immunological repertoire by the semisynthesis of antibody genes. A large library of human antibody Fab fragments differing only in the HCDR3 was displayed on the surface of phage and selected for antigen specificity. This method provides a route for generation of combinatorial antibody libraries where the starting repertoire is naive and of a size that at least equals that displayed by nature.

MATERIALS AND METHODS

Reagents and Strains. Restriction endonucleases were obtained from Boehringer Mannheim, *Taq* polymerase was from Promega, 7-deazadeoxyguanine 5'-triphosphate reagent kit and Sequenase version 2.0 were from United States Biochemical, T4 DNA ligase was from BRL, and oligonucleotides were from Research Genetics (Huntsville, AL) and Operon Technologies (Alameda, CA). *Escherichia coli* XL1-Blue and VCSM13 were obtained from Stratagene.

Construction of a Heavy Chain Fd Gene Randomized in CDR3. The human tetanus toxoid-binding Fab 7E (4) constructed in the pComb 3 vector (3), pC3TT7E, was used as a template for the PCR. Two PCRs were performed. The mixture for reaction 1 consisted of 10 ng of template pC3TT7E, 1 μg of primer B7EFR3 (5'-TCT-CGC-ACA-ATA-ATA-CAC-GGC-3'), 1 μg of primer FT3X (5'-G-CAA-TTA-ACC-CTC-ACT-AAA-GGG-3'), all four dNTPs (each at 200 μM), and Promega *Taq* polymerase buffer containing 1.5 mM $MgCl_2$ and 5 units of *Taq* polymerase in a final volume of 100 μl. Reaction 2 was identical to reaction 1 except that the reaction mixture contained primers CGIz (4) and 7ECDR3 [5'-GTG-TAT-TAT-TGT-GCG-AGA-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-TGG-GGC-CAA-GGG-ACC-ACG-3', in which N is A, C, G, or T (equimolar) and S is G or C (equimolar)]. The PCR was performed by denaturing for 1 min

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Abbreviations: Fl-BSA, fluorescein isothiocyanate-labeled bovine serum albumin; CDR, complementarity-determining region; HCDR3, heavy chain CDR3; FR, framework region; Phabs, phage displaying antibody combining sites.

at 94°C, annealing for 1 min at 50°C, and extending at 72°C for 2 min for 35 cycles. The resulting PCR products were gel-purified. Finally, in PCR mixture 3, 100 ng of product 1 [≈400 base pairs (bp)] from reaction 2 were added as templates for overlap extension (5) with the addition of 1 μg of primer FTX3 and 1 μg of primer CG1z in a 100-μl reaction mixture as described above. The PCR was performed as described above. The resulting fragment (≈790 bp) was gel-purified and digested with the restriction enzymes *Xho* I and *Spe* I as described (6) and gel-purified to yield product 3, a heavy chain fragment randomized in CDR3.

Library Construction. The plasmid of pC3TT7E was digested with *Xho* I and *Spe* I and gel-purified as described (6). Ligation of product 3 with the prepared vector was performed at room temperature overnight by the addition of 2 μg of the vector and 640 ng of product 3 with 10 units of T4 DNA ligase and BRL ligase buffer in a total volume of 150 μl. Five such ligations were performed, and after incubation, the DNA was precipitated and introduced by electroporation into *E. coli* XL1-Blue as described (6).

Preparation of Phage and Selection of Fluorescein-Binding Phage Displaying Antibody Combining Sites (Phabs). Phage were prepared as described (3, 6), except that after addition of kanamycin, the culture was grown at 30°C overnight. Fluorescein isothiocyanate-labeled bovine serum albumin (Fl₁₇-BSA; 17 fluorescein molecules per protein) was coated on a microtiter plate and served as a selective surface for panning (3, 6). After washing, bound phage were eluted with an acidic buffer or with fluorescein in phosphate-buffered saline.

Where fluorescein was used to elute phage, phage were eluted with 10 μM fluorescein in the first round of panning. In subsequent rounds, phage were eluted with 1 μM fluorescein. Elution with fluorescein was performed by the addition of 50 μl of fluorescein solution per well followed by incubation at 37°C for 1 h. The solution was then vigorously pipetted up and down to wash the well and transferred to 2 ml of fresh *E. coli* XL1-Blue for infection and amplification (6).

Preparation of Fab and Selection of Reactive Clones. After the final round of panning, double-stranded vector DNA was isolated and the gene III portion was excised to allow for production of soluble Fab (3). Reactivity was determined by an ELISA (4).

Determination of Affinity and Specificity. Affinity to Fl-BSA was determined by a competitive ELISA as described (4, 7). Acid-eluted clones were studied using Fl₁₇-BSA and all other clones were studied using Fl₇-BSA.

For the determination of dissociation constants by fluorescence quenching, equilibrium binding studies were performed using an SLM Aminco (Urbana, IL) 500C spectrofluorimeter. Lamp output was calibrated daily with the water Raman band (323 nm when excited at 292 nm). Fluorescein was recorded with excitation set to 492 nm (2 nm) and emission set to 530 nm (4 nm). Fluorescein and Fab solutions were prepared in 50 mM potassium phosphate (pH 8.0) and calibrated by UV, employing $\varepsilon_{495,\text{pH } 8.0} = 80,000$ and $\varepsilon_{280,\text{ pH } 8.0} = 1.4$, respectively. Equilibrium dissociation constants (K_d) were determined for preparations of human anti-fluorescyl-Fab fragments by equilibrium fluorescence quenching assay as described (8). Fluorescence quenching data were analyzed using both Scatchard and Sips plots. Optimum antibody concentration {[Fab] = K_d = K_a^{-1} = 1/x-axis intercept of Sips plot (9)} was determined for each clone prior to final fluorescence quenching experiment.

Specificity of clones was examined by an ELISA on Costar 3690 microtiter plates. Antigens were coated at 0.1 μg per well at 4°C overnight, the plates were blocked with 3% (wt/vol) BSA for 1 h at 37°C, and an ELISA was performed as reported (4).

RESULTS

Library Construction. The human tetanus toxoid binding Fab clone 7E (4) was randomly selected for mutagenesis. Targeting of the HCDR3 was obtained by PCR. An oligonucleotide was synthesized that is complementary to 18 bases on both the FR3 and FR4 sides of CDR3 with a randomized sequence in the central 48 bases corresponding to the actual CDR. PCRs with this oligonucleotide and one complementary to the 3′ end of the gene provided a gene fragment that encodes from the end of FR3 to the end of the Fd fragment. The other half of the gene was also produced by a PCR. These two products were then combined and fused in a final PCR (Fig. 1).

The final product, an antibody Fd fragment randomized in sequence in only the CDR3 region, was cloned into the pComb 3 vector containing the 7E light chain. The synthetic CDR3 can have about 10^{20} members and, thus, the diversity sampled is limited only by the number of transformants that can be studied. In the present case, transformation of *E. coli* with the plasmid DNA resulted in a library of 5×10^7 clones. Sequence analysis of DNA from the unselected library verified the targeted mutagenesis. Furthermore, the first two positions of the codons revealed an approximate equimolar distribution of bases whereas the third position was equimolar in guanine and cytosine.

Selection for Fluorescein Binding. Phabs were selected for binding to a microtiter dish coated with Fl₁₇-BSA. Specific Phabs were selected through four rounds of panning, elution, and amplification. The elution step was performed either with acidic buffer or by competition with free fluorescein. The acidic-buffer regimen resulted in the elution of 5.6×10^5, 4.6×10^6, 3.8×10^5, and 1.3×10^6 phage per well during the course of selection where input phage were approximately equivalent in titer, 1×10^{11}. Elution with free fluorescein yielded 4.7×10^5, 5.6×10^5, 1.4×10^6, and 4.0×10^6 phage per well, respectively, during the course of selection. Nonspecific binding to this surface with control phage varied between 10^4 and 10^5 phage per well. Production of soluble Fab and verification of binding by an ELISA revealed 8 reactive clones of 60 and 38 reactive clones of 40 for the acid-eluted and fluorescein-eluted libraries, respectively.
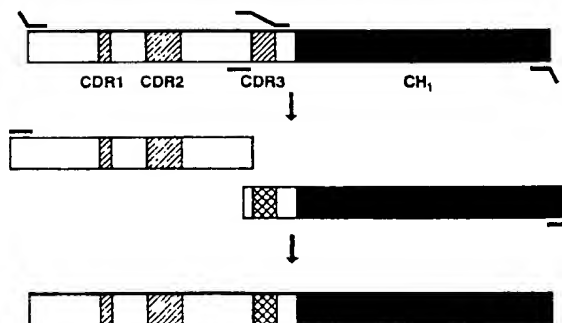

FIG. 1. Construction of the Fd fragment randomized in CDR3. The human anti-tetanus toxoid antibody clone 7E was used as a template for PCR. A PCR with primers FTX3 and B7EFR3 produced a gene fragment encoding a sequence from FR1 to FR3 (FR1 precedes CDR1). A PCR with primers 7CDR3 and CG1z produced a gene fragment encoding a sequence from the last 18 bp of FR3 to the end of the Fd portion of the heavy chain and introduced the (NNS)₁₆ sequence into the CDR3. Fusion of these two products in a final PCR with primers FTX3 and CG1Z utilizes the last 18 bp of the first product, which are identical to the first 18 bp of the second product, as an overlap region (5). Open boxes, framework regions; hatched boxes, native CDR regions; cross-hatched boxes, randomized CDR region; solid boxes, CH₁ region.

Sequence Analysis. The complete nucleotide sequence of the mutated heavy chain of a representative number of Fl-BSA binding clones was determined. No PCR-induced mutations outside of the CDR3 were observed. The predicted amino acid sequences of the HCDR3 are shown in Fig. 2. Clones recovered from the acid-elution regimen show no consensus sequence. Conversely, the clones isolated from the fluorescein-elution regimen show remarkable selection of consensus sequences. Of 10 clones sequenced, only 3 sequences were observed. All of these clones had glycine at position 95 and aspartic acid at position 101 (Kabat numbering system) (10). Both possible glycine codons provided by the synthesis were used. Furthermore, 9 of the 10 clones contained a Ser-Arg-Pro sequence near the center of the loop directly adjacent or one residue removed on the N-terminal side of an arginine residue, though the codon usage for two of these residues was different. Clone F31 lacks this central motif. The finding that different codons were used provides strong support for the proposition that clonal selection occurred at the level of antigen–antibody union and not because of some unexpected bias of nucleotide incorporation into DNA.

Characterization of Specificity and Affinity. Clones were selected for reactivity with the highly labeled Fl$_{17}$-BSA conjugate. However, only those clones that were obtained in the fluorescein-elution regimen were highly reactive with a conjugate with a lower level of fluorescein labeling, Fl$_7$-BSA. Since the original clone 7E bound tetanus toxoid with a K_d of approximately 0.1 μM, it was of interest to determine the reactivity of the mutated clones with this parent antigen. As shown in Fig. 3, the reactivity profiles of the mutant clones are quite different from the parent, demonstrating a clear shift in specificity. Clones from the acid-elution regimen were more highly cross-reactive with the parent antigen tetanus toxoid (data not shown).

Affinities for the labeled conjugate were determined by a competitive ELISA and representative results are shown in Fig. 4. All clones had approximate K_d values in the range of 0.01–0.1 μM for the conjugates Fl$_7$-BSA and Fl$_{17}$-BSA for the fluorescein- and acid-elution regimens, respectively. No competition of the parent clone 7E for Fl$_7$-BSA was noted in the concentration range examined (10^{-10} to 10^{-5} M), suggesting an affinity of <10 μM.

True K_d values were determined in fluorescence quench assays (8, 9). Representative results of the Scatchard and Sips plots for two of the five clones are shown in Fig. 5. Clones
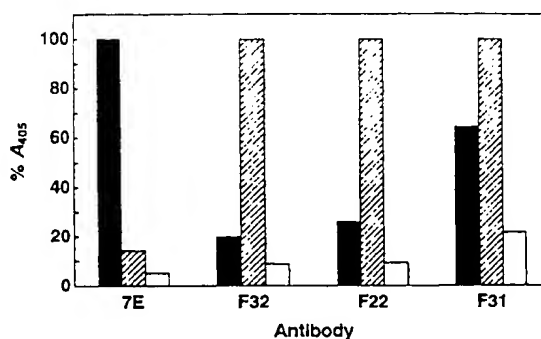
FIG. 3. Comparison of the binding specificity of the starting clone with several Fl-BSA-selected clones. Specificity was examined by an ELISA. Antigens: solid bars, tetanus toxoid; hatched bars, Fl$_7$-BSA; open bars, BSA.

from the fluorescein-elution scheme have higher affinities for fluorescein, 0.1 μM versus 1 μM for the acid-eluted clones. The parent clone 7E showed no quenching within the detectable limits of the assay, suggesting an affinity for free fluorescein of <10 μM. Binding constants have been compiled adjacent to the appropriate sequence in Fig. 2.

DISCUSSION

In the past, access to the immunological repertoire has been limited. The development of the combinatorial approach to antibody cloning (1) has allowed unrestricted access to the immunological repertoires of both mice and humans. Indeed, every compartment of the natural B-cell repertoire has yielded antibodies by the combinatorial approach. Attention was initially focused on the affinity-matured antibodies that could be obtained from immunized mice (1, 11, 12) and humans (4, 13) or in special cases from infected individuals for the cloning of anti-viral antibodies (14). The memory compartment has been accessed by transfer of human peripheral blood lymphocytes after antigen stimulation into severe combined immunodeficiency mice (15) and the naive repertoires of mice and humans have been probed with some success (16, 17). Finally, for mouse, the process of affinity maturation by somatic mutation has been mimicked by PCR mutagenesis and phage selection (16).

FIG. 2. Amino acid sequences of the CDR3 of the starting and selected clones and their affinities for free fluorescein (Fl) and Fl-BSA. *, Approximate K_d value as determined by a competitive ELISA.
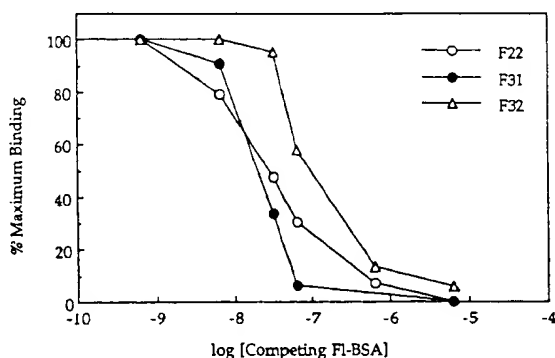
FIG. 4.   Competitive ELISA for the determination of approximate K_d values of Fabs for Fl-BSA.

Access to much larger naive and structurally diverse antibody libraries is an important part of solving problems of recognition and catalysis. The mouse recognizes a seemingly unlimited number of antigens while displaying a naive repertoire of less than 10^8 members (18). The naive repertoire available to the mouse (18) is far greater than that which it displays at a given moment. Access to naive repertoires of animals can be provided by PCR and cell sorting techniques; however, the character of this repertoire will be influenced or edited by the animal and will be extremely susceptible to contamination from materials derived from activated B cells or plasma cells, which would limit the diversity of the library.

A chemical solution to this diversity problem can be achieved by semisynthesis or total synthesis of antibody
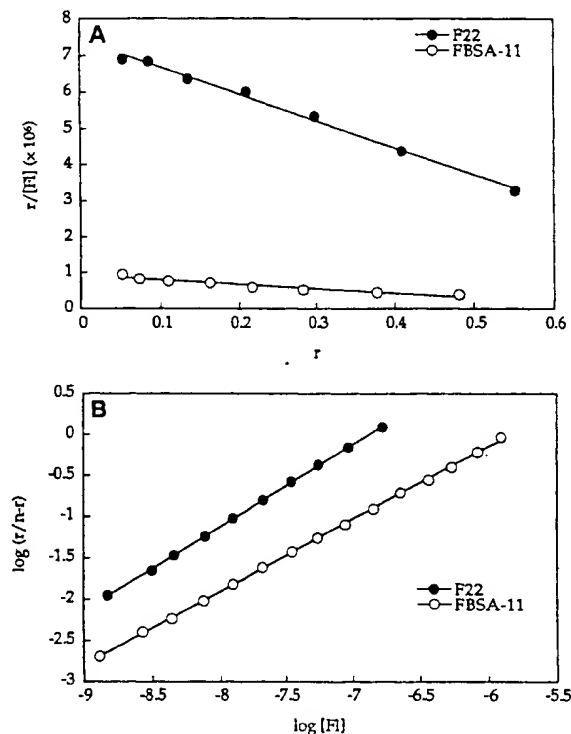
FIG. 5.   Equilibrium binding parameters were obtained by Scatchard (*A*) and Sips (*B*) analyses of fluorescence quenching data. Representative results for two of the five Fabs are shown above. Fl, fluorescein; r, mol of antibody-bound fluorescein per mol of antibody; n, antibody valence.

genes wherein diversity is controlled by the oligonucleotide synthesis. In this first experiment, we have focused on randomizing the HCDR3 sequence of a single clone, as HCDR3 is the most hypervariable region in an antibody molecule. It has recently been estimated that humans have the potential to generate as many as 10^{14} peptide sequences in this region (19). The second reason HCDR3 was targeted is that HCDR3 makes the largest contribution to the total accessible surface area of an antibody combining site (20).

The 16-amino acid sequence of the 7E HCDR3 was randomized utilizing PCR to incorporate $(NNS)_{16}$ to encode the 16 random residues. NNS encodes all 20 amino acids and a single stop codon TAG, which is suppressible in the *supE E. coli* strain employed in these studies. The possible number of peptide sequences produced from this synthesis exceeds 10^{20} though the library contained only 5×10^7 transformants, which is similar in size to the repertoire of a mouse. This characteristic of displaying only a fraction of the available diversity is also similar to nature's strategy. However, in semisynthetic libraries one can select for more specificities by simply generating additional transformants. This is equivalent to the much more cumbersome process of studying more animals if a desired antibody was not obtained in the initial immunization.

Antibody Fab fragments were displayed on the surface of phage (Phab) by using the monovalent display vector pComb 3 that allows for the rapid sorting of the highest-affinity Fab fragments (3). Phab libraries were sorted by antigen elution or acid elution. Elution with fluorescein gave more consistent enrichments during the panning process presumably by providing a more selective enrichment for fluorescein binding over nonspecific binding. Furthermore, elution with the hapten provides selective pressure to enrich for the antibodies that make most of their binding contacts with the hapten versus the hapten–protein conjugate. Indeed, the affinities of the fluorescein-eluted antibodies for the conjugate and for free fluorescein are more closely matched than those obtained from the acid-elution regimen.

The affinities of the best antibodies obtained in this study (0.1 μM) approach the average K_d values of the secondary response of immunized mice for free fluorescein, which is in the order of 0.1 μM (21). Furthermore, these affinities, 0.1–1 μM, compare favorably to antibodies cloned from phenyloxazolone-conjugate-boosted mice, where the average observed affinity is 1–10 μM (12). The specificity of the selected clones was greatly perturbed compared to the starting clone, effectively changing a tetanus toxoid binding antibody into a fluorescein binding antibody.

A unique feature of the clones obtained using the fluorescein-elution regimen is their striking similarity in sequence. All of these clones contain glycine at position 95 encoded by both of the two possible codons and aspartic acid at position 101 encoded by the only codon provided by the synthesis protocol, which in all produces 32 possible codons. In natural antibodies, Asp-101 usually plays a structural role by forming a salt-bridge with Arg-94 in FR3 (20). Thus, the artificial selection process has recapitulated an interaction of structural significance that in the animal resulted from the process of natural selection.

The other striking feature of the selected semisynthetic antibodies is the appearance of the Ser-Arg-Pro sequence in the central position adjacent or one residue removed from an arginine. Though these three amino acids are overrepresented by the synthetic strategy, their chance occurrence adjacent to each other in the sequence and in similar loop positions is highly unlikely. All sequences are rich in arginine, which is encoded by 3 of the 32 possible codons. Comparison of the occurrence within the 10 CDR3 sequences of arginine with leucine and serine, which are also encoded by three possible codons in the synthesis, reveals an Arg/

Leu/Ser ratio of 29:16:15. This bias toward selection of arginine may be a result of the dianionic character of fluorescein. The crystal structure of a fluorescein–Fab complex has implicated involvement of arginines in charge neutralization (22). In this structure, arginine is sufficiently close to one enolic group on the xanthonyl ring of fluorescein for electrostatic interaction. The lack of consensus behavior in the acid-eluted clones may be contributed by their recognition of a more complex epitope consisting of fluorescein and BSA and is reflected in the more disparate affinities to fluorescein and Fl-BSA.

Linear peptide epitope libraries have been probed to identify alternative ligands for receptors (23, 24). The construction presented here could be regarded as a random-sequence generator for synthesis of peptide loops. Such structured loops might be more easily translated into non-peptidyl ligands for receptors and may be of use in drug design. The success of this type of strategy cannot be easily predicted since loop variation may serve to optimize binding contacts elsewhere in the antibody but the large region of conformational space that can be sampled is cause for some optimism.

Semisynthesis of antibody genes is a step in the direction of totally synthetic antibodies. The diversity of semisynthetic libraries might be most effectively increased by combining CDR3 libraries with those of natural gene fragments from FR1 to FR3 or by synthesizing all the CDRs. These approaches should offer a route to immunoglobulin diversity thereby providing antibodies for therapy and catalysis.

Note Added in Proof. Clones derived from the HCDR3 library selected for binding to fluorescein have been combined with a light-chain library randomized in the CDR3 region and reselected for binding to fluorescein. Again, consensus behavior was observed in the selected clones. Furthermore, at least three light-chain nucleotide sequences coding for the same amino acid sequence were obtained.

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